Gallic acid and its esters will suppress the contractile response elicited from the smooth muscle of the guinea pig ileum by the action of bradykinin. The initial suppressive effect of members of a homologous series of gallates increases almost directly with chain length. Plots of reciprocal of gut response against the reciprocal of bradykinin concentration for different concentrations of these food grade antioxidants indicate that they might act as competitive inhibitors. The mechanism of their inhibitory action is apparently complex and, in the case of the longer chained esters, is only partially reversible.

Bradykinin is a vasoactive peptide whose physiological role in living systems has undergone considerable investigation since its presence was first reported in human blood plasma (Roche e Silva *et al.*, 1949). Some organic compounds related to tranquilizers antagonize the bradykinin induced smooth muscle contractions of isolated guinea pig ileum (Roche e Silva and Garcia Leme, 1964). The type of drug inhibition exhibited by some of these chemicals has been characterized (Garcia Leme and Roche e Silva, 1965).

During the course of our research we noted that some common food grade antioxidants could likewise suppress smooth muscle response to bradykinin. Because antioxidants of this type are employed for food preservation, we report here results obtained during an investigation into the nature of the inhibition of guinea pig ileum response effected by gallic acid and a homologous series of its esters.

MATERIALS AND METHODS

Assays were conducted using a 2- to 3-cm section of the terminal segment of the guinea pig ileum which was suspended in a 5-ml capacity perfusion bath held at 37° C. A steady stream of air was bubbled through the reaction chamber for stirring and oxygenation of the ileum preparation. The ileum was attached to a frontal lever pen which recorded change in length on a kymograph. Before being used for analytical work, the gut was equilibrated for 1 hr in a carrier solution consisting of Tyrode's buffer containing 2 mg per liter of atropine and 40 μg per liter of pyribenzamine. Stock solutions of synthetic bradykinin (BRS 640, Sandoz Pharmaceutical Co.) were prepared in Tyrode buffer. Control responses to the kinin were determined by injecting 0.2 ml of the stock solution into the reaction chamber after buffer flow had been interrupted. After a 30-sec exposure of the smooth muscle to the drug, the cell was flushed by buffer at a rate of 15 ml per min. The height of the peak produced on the kymograph was taken as a direct measurement of the amount of smooth muscle contraction produced by bradykinin. Control responses to histamine dihydrochloride (Nutritional Biochemicals Corp.) were established.

Assay in the Presence of Inhibitors. The same conditions as in the previous assay were adhered to. Stock solutions of gallic acid (Fisher Chemical Co.), ascorbic acid (Fisher Chemical Co.), and the gallate esters, methyl, ethyl, *N*-propyl, isopropyl, *N*-butyl, *N*-amyl, and *N*-hexyl, prepared according to the procedure of Russell and Tebbens (1942), were made up in distilled water. Suitable concentrations of these antioxidants were mixed with varying concentrations of bradykinin immediately before injection into the bath. Decreases in peak heights effected by the presence of the antioxidants in the test mixtures were used to evaluate their inhibitory action.

Computation of Data. All concentrations are presented as concentrations in the reaction bath.

The nature of the inhibition of the smooth muscle response to bradykinin, which these antioxidants produce, was determined according to the general method of Roche e Silva (1959). Results were interpreted by plotting reciprocals of effects ($1/Y$) *vs.* reciprocals of doses of bradykinin ($1/X$). Y is the height of the peaks produced on the kymograph chart as measured in mm and X is the concentration of bradykinin in μg/ml.

The inhibitory potency of the gallate esters was evaluated from these plots and expressed as the pK_i value. Roche e Silva has employed these double reciprocal plots to substantiate linearity between the number of receptors occupied by a drug and the effect as measured on a kymograph. The action of the drug is applicable to Clark's equation:

$$K_c X = \frac{Y}{1 - Y} \tag{1}$$

where X is the concentration of drug in g/ml, Y the response obtained on the chart, and K_c is Clark's constant. If the straight line obtained when such a plot is normalized, that is, the Y intercept or maximum drug response is taken as 1, and the responses taken as percentages of the maximum response, the slope of this line K_n is the reciprocal of Clark's constant. Inversion of Clark's equation gives:

$$\frac{K_n}{X} = 1/Y - 1 \tag{2}$$

and pK_n is a measure of the affinity of the drug for the receptors.

Introduction of an inhibitor into the system follows Gaddum's equation:

$$K_c X = (1 + I/K_i)\frac{Y}{1 - Y} \tag{3}$$

where I is the concentration of inhibitor, K_c Clark's constant, and K_i a constant dependent on the nature of the inhibitor. Inversion of this equation gives:

$$\frac{\beta K_n}{X} = 1/Y - 1 \tag{4}$$

where β is $1 + I/K_i$.

The effect of the inhibitor is to change the slope of the

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normalized line by the multiple β without, in the case of competitive inhibition, changing the intercept. For competitive inhibition the pK_i value can be calculated from β according to the equation:

$$-pK_i = \log (I) - \log (\beta - 1) \quad (5)$$

pK_i is a measure of the affinity of the inhibitor for ileum receptor sites. Experimentally, β is the ratio of the slopes of the lines with inhibitor to the slope of the control line. In the case where β equals 2, the pK_i value is equivalent to a pA_2 value; that is, the concentration of the inhibitor reduces the effect of a double dose of drug to that produced by a single dose. The pK_i values presented here are calculated from the slopes of lines where β was as close to 2 as was possible to attain experimentally.

RESULTS AND DISCUSSION

The threshold levels at which gallic acid and its six homologous esters begin to produce an inhibition of ileum response to 0.04 μg/ml bradykinin is related to the number of carbons in the ester side chain (Figure 1). As the chain length of the acid substitution group increases, the kinin inhibitory power of these antioxidants also increases. The only deviation from this pattern is with hexyl gallate. No difference is noted in the repressive strength of the isomeric forms of propyl gallate.

The observed suppressive effect of the gallates cannot be ascribed to some impurity in the preparations, since all the esters, except hexyl, were made from chromatographically pure alcohols, and all melted at values reported in the literature. Furthermore, the esters migrated as a single spot when analyzed by thin-layer chromatography.

The effect of increasing levels of the phenolic antioxidants on the muscular response to 0.04 μg/ml bradykinin is related to the chain length for gallic acid and the methyl, ethyl, and propyl derivatives (Figure 2). As the chain length is increased to 3 carbons, the change in concentration of antioxidant required to significantly depress gut response becomes greater. When high concentrations of these esters were employed in the reaction mixture, the ileum did not return quickly to normal kinin sensitivity. Flushing of the cell for a period of 30 min was required to produce a normal response to the control after application of propyl gallate in an amount sufficient to completely suppress gut response to bradykinin.

The effect of chain length on the muscle response becomes less obvious as it is increased beyond 3 carbons (Figure 3). The results presented in this figure are complicated by the fact that the ileum never returned to normal kinin sensitivity after exposure to high levels of these compounds and after repeated doses of low levels. The raw data were corrected for loss in kinin sensitivity and are presented as percent suppression of responses to control doses run within 5 to 10 min after flushing the cell of the previous reaction mixture.

To establish the nature of the bradykinin inhibition of the gallate esters, a drug dose study was conducted. A typical double reciprocal plot (Figure 4) shows inhibition of gut contractions in systems containing fixed levels of antioxidant as the kinin dose is decreased. The relatively straight lines passing through a common point on the Y axis are typical of those obtained when a competitive inhibitor is influencing a biological system. Similar curves were obtained for methyl and ethyl gallate and for the higher members of the homologous series at low concentrations. In those instances where repeated small doses of the butyl, amyl, and hexyl gallates decreased the ileum's normal kinin sensitivity, the data were

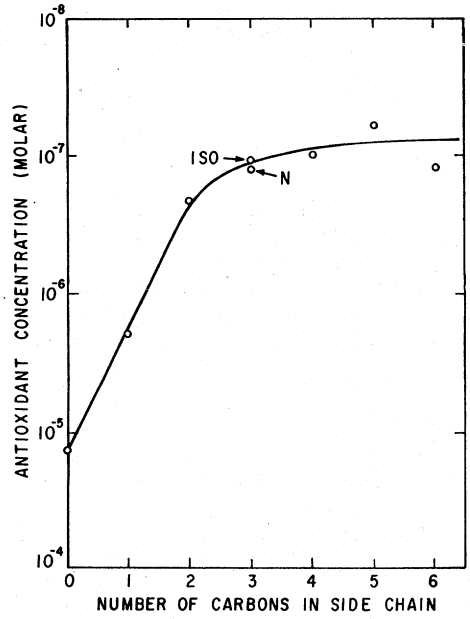

Figure 1. Minimum antioxidant concentrations required to detectably inhibit ileum response to 0.04 μg/ml bradykinin

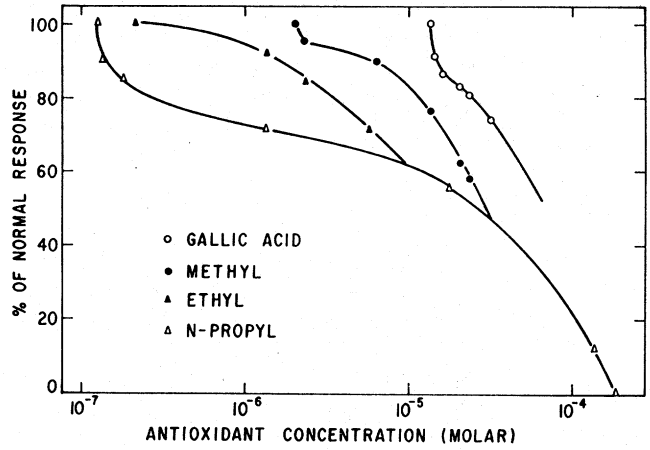

Figure 2. Suppression of ileum response to test solutions of bradykinin containing increasing levels of gallic acid, methyl, ethyl, and *N*-propyl gallate

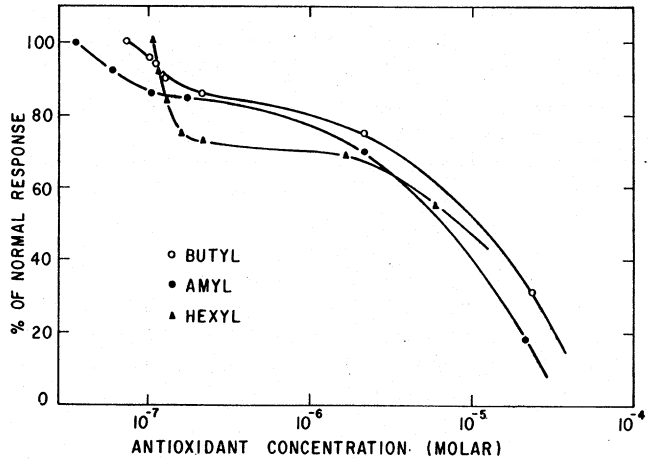

Figure 3. Suppression of ileum response to test solutions of bradykinin containing increasing levels of butyl, amyl, and hexyl gallate

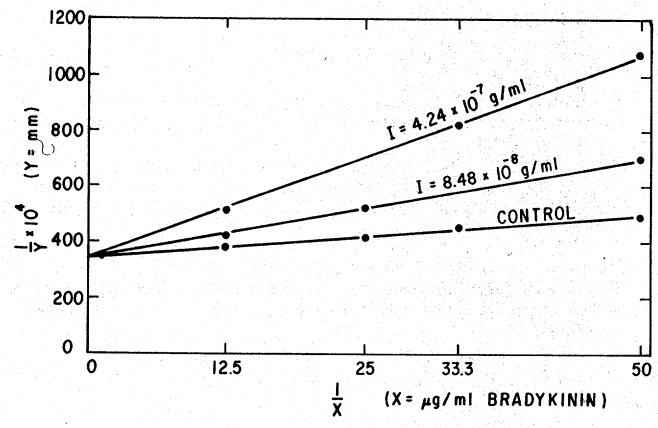

Figure 4. Drug dose (X)-response (Y) plot for N-propyl gallate

I = gallate concentration

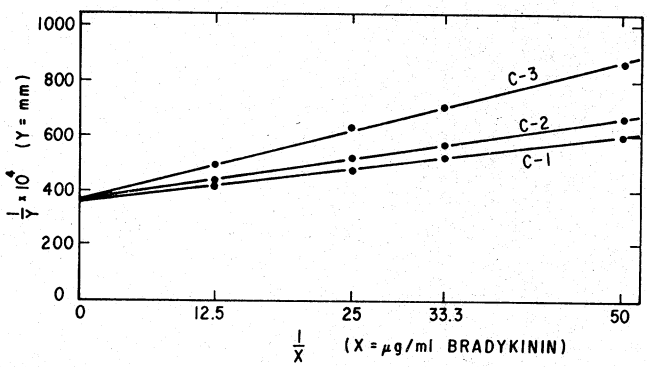

Figure 5. Drug dose (X)-response (Y) plot of control values for bradykinin

Points on line C-1 are original responses
Points on line C-2 are obtained after first series of tests with butyl gallate
Points on line C-3 are obtained after 2nd series of tests with butyl gallate

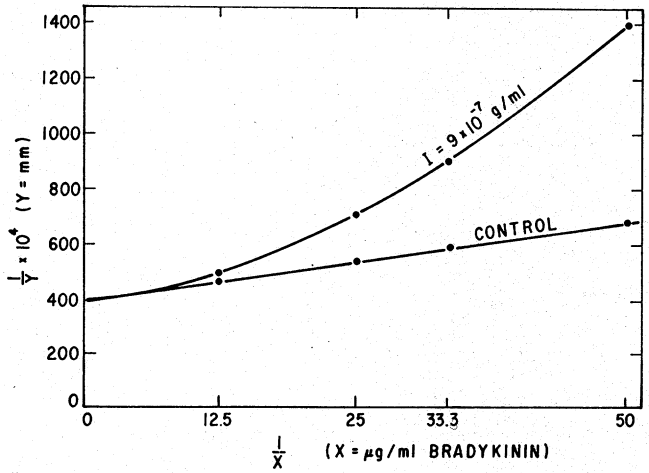

Figure 6. Drug dose (X)-response (Y) plot for butyl gallate

I = gallate concentration

corrected for change in gut response to the kinin standard, and the straight line plots were always obtained.

pK_i values (Table I), calculated from the slopes of the experimental lines, show methyl and ethyl gallate to be relatively poor inhibitors of bradykinin. The other antioxidants are relatively strong inhibitors.

Table I. pK_i Values (w/v) of Gallate Esters

Gallate Ester	pK_i(w/v)
Methyl	4.12
Ethyl	6.00
N-Propyl	7.10
iso-Propyl	7.06
Butyl	7.07
Amyl	7.02
Hexyl	6.80

The decrease in smooth muscle sensitivity effected by butyl, amyl, and hexyl gallate is only partially reversible (Figure 5). Control values for all bradykinin doses are decreased by repetition of doses of these esters. The time for recovery to a stable response, after addition of samples, lengthens with their number and concentration. Once this irreversible effect sets in, control responses do not return to normal.

When high concentrations of all the antioxidants are employed, the straight line relationships between the reciprocals of doses and responses are not obtained (Figure 6). Deviation from linearity is most conspicuous with low kinin doses, and occurs with concentrations of gallate sufficient to more than double the slope of the control line. This effect is most noticeable beyond the propyl derivative.

The inhibitory action of the antioxidants studied is complex. From the graphical data, the esters appear to exhibit competitive drug antagonism. However, it is not a classical competitive antagonism whereby small changes in antioxidant concentration produce marked changes in the slopes of the reciprocal dose effect curves. Large changes in antioxidant concentration are required to turn the angle of the line with inhibitor to produce any significant changes in β.

The inhibitory effects of the methyl, ethyl, and propyl derivatives of gallic acid are largely reversible. Irreversible changes occur in the gut tissue when exposed to the higher members of this series. These higher gallates may decrease gut sensitivity by binding strongly or irreversibly to sites on or near those utilized by the kinin. It is doubtful that the higher homologs change bradykinin sensitivity by destroying the general cell function, because full response of gut tissue to histamine occurs in the presence of all the antioxidants tested up to 5×10^{-4} M concentration.

We are aware of the fact that these antioxidants are slowly oxidized in distilled water and more rapidly oxidized in basic solutions producing detectable spectral changes. Fresh stock solutions of these compounds were therefore prepared every several days and kept refrigerated to insure their stability.

To determine whether there was any chemical interaction between the antioxidants and bradykinin prior to addition of the mixture into the cell, the effect of the sequence of the addition was examined. No differences in inhibitory action were noted between injection of the antioxidants followed by iniection of bradykinin and injection of the mixture of the two materials. In one experiment a low concentration of methyl gallate was introduced into the Tyrode buffer supply and doses of bradykinin were injected into the bath. The results were the same as when the mixture of the two compounds were simultaneously injected into the bath containing only Tyrode buffer.

The observed inhibition may result from the presence of phenolic groups in the molecule which give rise to directed binding. The reducing capacity of the molecules is probably not involved, as ascorbic acid, another antioxidant, does not

inhibit smooth muscle contraction produced by bradykinin. The contractile action of this physiologically active peptide is definitely inhibited by gallic acid and six of its homologous esters, one of which is an important food grade antioxidant. The significance of these studies in terms of the usage of gallates for food preservation remains unknown.

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